LESSON 22: PLANT TISSUE CULTURE- AN ALTERNATIVE FOR SECONDARY METABOLITE PRODUCTION

Objective

Higher plants contain a variety of substances which are useful medicines, food additives, perfumes, etc. However, decreased plant resources, increases in labour cost and other problems in obtaining these high-value added substances from natural plants have pointed toward the use of plant cell culture for production of the products. Because plant cell culture is not affected by changes in such environmental conditions such as climate or natural degredation, improved production may be available in any place or season.

Therefore, studies on the production of useful metabolite by plant cell culture have been carried out on an increasing scale since the end of the 1950's. The large scale cultivation of tobacco and various vegetable cells was examined in the late 1950's and early 1960's in the U.S., Canada, and Europe. Their results stimulated more recent studies on the industrial application of this technology in many countries.

Since plant cells can be cultivated in various fermentors in a similar way to microbial fermentations, many industrial companies in Japan have tried to apply this technology to commercial production of useful compounds because Japan has a highly developed fermentation technology. At present, two Japanese firms are manufacturing a plant pigment, shikonin, and ginseng cell biomass on a commercial scale and several other products including anti-cancer drugs seem to be close to commercialization. However, there are still a few barriers which must be overcome before commercialization of many other products can occur. The production cost of metabolites is one of the problems because of the low productivity of cultured plant cells. In order to decrease the cost, increase of production efficiency per cell is an essential factor, which means that higher amounts of products must be produced as quickly as possible. A variety of approaches to improve the productivity of the culture have been tried and some of them were very effective.

In this Unit, the background of plant cell culture research, methods and facilities, various approaches to improve the productivity, and studies on production of a number of commercially interesting products which have currently been investigated are described.

Introduction

Plant cell culture is viewed as a potential means of producing useful plant products such that conventional agriculture, with all its attendant problems and variables, can be circumvented. These problems include: environmental factors (drought, floods, etc.), disease, political and labour instabilities in the producing countries (often Third World countries), uncontrollable variations in the crop quality, inability of authorities to prevent crop adulteration, losses in storage and handling. Thus, the production of useful and valuable secondary metabolites in large bioreactors located in the consuming country is an attractive proposal. Additional advantages of such processes include: controlled production according to demand and a reduced timerequirement.

However, this technology is still being developed and despite the advantages outlined above, there are a variety of problems to be overcome before it can be adopted on a wide scale for the production of useful plant secondary metabolites. The success of Mitsui Petrochemical Industry Co. Ltd. in Japan in producing shikonin on a commercial scale from Lithospermum erythrorhizon cultivations and that of Nitto Denko Co. Ltd. also in Japan in mass production of Panax ginseng or ginseng cells using 20 kL tanks have demonstrated that many of the problems can be overcome with perseverance. The economic feasibility of these processes is another question and this will be dealt with in a separate section.

In theory, it is anticipated that such large scale suspension cultures will be suitable for industrial production of useful plant chemicals such as pharmaceuticals and food additives, in a manner similar to that of microbial fermentation.

Nevertheless, there are some significant differences between microbial and plant cell cultures that must be considered when attempting to apply plant cell cultures to the available technology. Table.1 shows a comparison of some of the characteristics of plant and microbial cultures of relevance to fermentation. This table serves to demonstrate some of the problems that can be encountered with plant cell cultures. The sensitivity to shear is due both to the large size of the cells and to the relatively inflexible cellulose cell wall. Thus, with normal blade impellers the cells may twist which will inhibit mitoses and, for this reason, air- lift fermentors are recommended by some researchers. The large size of the plant cell contributes to its comparatively high doubling time (12 h - several days), which thus prolongs the time required for a successful fermentation run.

The vacuole is the major site of product accumulation, and since product secretion is uncommon, the high metabolite yields seen in microorganisms that secrete product (thereby removing product inhibition of biosynthesis) cannot be expected. There is some ongoing research on membrane permeabilization of plant cells which may serve to relieve the constraints of product inhibition by facilitation of leakage into the extracellular medium. If this would also permit recycling of the biomass (e.g. via immobilization) it would help reduce production costs.

The low aeration requirement for plant cells is an advantage over microbial cultures in general. In addition, the high cost of running a fermentation vessel over several weeks should be considered, although media costs are much less than those of animal cell cultures.

Table.1.
Characteristic of Microbial and Plant Cell Relevant to Fermentation

Characteristics	Microorganism	Plant Cell
Size	2 u	>10 u
Shear stress	Insensitive	Sensitive
Water content	75%	>90%
Duplication time	<1 hour	days
Aeration	1-2 vvm	0.3 vvm
Fermentation time	Days	Weeks
Product accumulation	Medium	Vacuole
Production phase	Uncoupled	Often growth-linked
Mutation	Possible	Requires haploids
Medium cost ($) (MS medium)	8-9/m	65-70/m

Source: Zenk, M.H., Plant Cell Culture Conference, Oyez Sci, Tech. Serv. (1982)

In addition to the problems outlined above, concerned with fermentation technology, there are also considerable hurdles to be overcome at the biochemical level. The two major problems concern poor expression of products and instability of cell lines.

Cultured plant cells often produce reduced quantities and different profiles of secondary metabolites when compared with the intact plant and these quantitative and qualitative features may change with time. The poor product expression is often attributed to a lack of differentiation in cultures. On the other hand, there are cases of cultures that over-produce metabolites compared with the whole plant (Table 2).

There are a number of examples of cultured cells producing metabolites not observed in the plant, eg. Lithospermum erythrorhizon cultures have been observed to synthesize rosmarinic acid. It has become apparent that the choice of original plant material having high yields of the desired phytochemical may be important in establishing high-yielding cultures. Furthermore, the need to repeatedly screen for high-producing lines (due to inherent instability of cell lines) has been emphasized, although the nutritional composition of the medium is also important. Thus, a variety of approaches are being investigated by many researchers to increase productivity of useful plant metabolites in plant cell cultures as seen in another section in this Lesson.

Table 2.
Secondary Metabolites Produced in High Levels by Plant Cell Cultures

COMPOUND	PLANT SPECIES	YIELDS (% DRY WT)		CULTURE TYPE*
		CULTURE	PLANT	
Shikonin	Lithospermum erythrorhizon	20	1.5	s
Ginsenoside	Panax ginseng	27	4.5	c
Anthraquinones	Morinda citrifolia	18	0.3	s
Ajmalicine	Catharanthus roseus	1.0	0.3	s
Rosmarinic acid	Coleus blumeii	15	3	s
Ubiquinone-10	Nicotiana tabacum	0.036	0.003	s
Diosgenin	Dioscorea deltoids	2	2	s
Benzylisoquinoline Alkaloids	Coptis japonica	11	5 - 10	s
Berberine	Thalictrum minor	10	0.01	s
Berberine	Coptis japonica	10	2 - 4	s
Anthraquinones	Galium verum	5.4	1.2	s
Anthraquinones	Galium aparine	3.8	0.2	s
Nicotine	Nicotiana tabacum	3.4	2.0	c
Bisoclaurine	Stephania cepharantha	2.3	0.8	s
Tripdiolide	Tripteryqium wilfordii	0.05	0.001	s
* s = suspension; c = callus				

Historical Background

The production of plant metabolites by callus and cell suspension cultures has been carried out on an increasing scale since the end of the 1950's. The large scale cultivation of tobacco and a variety of vegetable cells was examined from the late 1950's to early 1960's by Tulecke and Nickell at Pfizer Inc., Mandels et al. at the Natick Laboratories in the U.S. Army, Street et al. at the University of Leicester and Martin et al. at the National Research Council of Canada. Their results stimulated more recent studies on the industrial application of plant cell culture in many countries.

Since Japan has a highly developed fermentation technology, many industrial companies, in collaboration with some university groups, have tried to apply this technology for the

commercial production of useful compounds. The Japan Tobacco Inc.'s interest involved around mass-production of tobacco cells as raw materials of cigarettes; the company established 20 kL fermentors which were the largest for plant cells in 1970's. Meiji Seika in Japan also elucidated the fundamentals of production of Panax ginseng cells in large volumes. Researchers reported that cultured ginseng cells stimulated physiological activities in animals in a similar fashion as elicited by native ginseng roots. The work was followed by Nitto Denko Co. which has been manufacturing cell mass of ginseng commercially. The cells are used as health foods in Japan. Researchers of Kyowa Hakko conducted extensive pharmacological screening of numerous cell cultures and found various novel products of great interest, including plasmin inhibitory proteins in Scopolia japonica cells and plant virus inhibitors in cultured cells of Phytolacca americana and other species. The virus inhibitors of P. americana are now being studied by many research groups in the world because of their activity against AIDS and other animal viruses. Other firms such as Ajinomoto and Nippon Shin-yaku also made efforts to increase the level of accumulation of alkaloids, steroids and other secondary products in cultured cells.

Groups in Germany outlined very interesting approaches to industrial application in the meeting held in 1976 at Munich, and their excellent results encouraged researchers in other countries. For example, Zenk and his colleagues presented an impressive paper in which they successfully selected high-alkaloid producing cell lines of Catharanthus roseus using a method similar to the microbial mono-colony isolation technique. A number of laboratories in industries and universities followed Zenk's approach, and in fact, some researchers could increase significantly the level of secondary products such as ubiquinone-10, biotin, and various plant pigments produced by cell cultures.

In 1982, the 5th International Congress of Plant Tissue and Cell Cultures was held in Japan and about 70 out of 372 papers presented there related to production of secondary metabolites in cultured cells and several papers seemed to be commercially promising such as production of shikonin by Fujita et al. of Mitsui Petrochemical and that of several antitumor compounds by Misawa et al. of Kyowa Hakko.

At subsequent International Congress of Plant Tissue and Cell Cultures in Minneapolis in 1986 and that in Amsterdam in 1990 as well as other meetings such as the Meeting of Primary and Secondary Metabolism of Plant Cell Cultures held in Giessen, Germany and the 4th and International Congress on Phytotherapy held in Munich, Germany in September, 1992, many compounds were shown to be accumulated by plant cell cultures and many different strategies were presented to increase their productivity. Means for production of those compounds include not only de novo synthesis but also biotransformation processes. A biotransformation process to produce ß-methyl digoxin using Digitalis lanata cells studied by Reinhard and Alfermann in Germany was evaluated by Boehringer Mannheim Co. using 4 kL bioreactors although it has not yet been commercialized.

A combination of a plant cell culture process and a simple chemical coupling reaction was invented by a Canadian company, Allelix, to manufacture vinblastine as a commercially feasible process. The technology is now being studied by Mitsui Petrochemical in Japan for commercialization.

Sanguinarine production was also studied by Kurz et al. in Canada since this alkaloid has a market in the denatal care field. The recent biotechnology boom has triggered increase interest in plant cell cultures, for example, a number of firms and academic institutions in the U.S., Japan, Canada, and Europe have been investigating intensively the production of a very promising anti-tumor compound, taxol, using this technology.

Materials And Methods

Materials

Plants

In theory, any part obtained from any plant species can be employed to induce callus tissue, however the successful production of callus depends upon plant species and their qualities. Dicotyledons are rather amenable for callus tissue induction, as compared to monocotyledons; the callus of woody plants generally grow slowly. Stems, leaves, roots, flowers, seeds and any other parts of plants are used, but younger and fresh explants are preferable as explant materials.

Explants obtained must be sterilized using ethanol, sodium hypochlorite and/or other chemicals to remove all microorganisms from the materials and a typical sterilization procedure will be described later as an example.

Media

Inorganic Salts

To induce a callus from an explant and to cultivate the callus and cells in suspension, various kinds of media (inorganic salt media) have been designed. Agar or its substitutes is added into the media to prepare solid medium for callus induction.

One of the most commonly used media for plant tissue cultures is that developed by Murashige and Skoog (MS) for tobacco tissue culture. The significant feature of the MS medium is its very high concentration of nitrate, potassium and ammonia. The B5 medium established by Gamborg et al.is also being used by many researchers. The levels of inorganic nutrients in the B5 medium are lower than in MS medium. Many other media have been developed and modified and nutrient compositions of some typical media will be described in Table3. However, it is not always necessary to test many kinds of basal media when a callus is induced. It would be better to use only one or two kinds of basal media in combination of different kinds and concentrations of phytohormones. The most suitable medium composition should be optimized afterwards in order to obtain higher level of products as well as higher growth rate.

Carbon Sources

Sucrose or glucose at 2 to 4% are suitable carbon sources which are added to the basal medium. Fructose, maltose and other sugars also support the growth of various plant cells. However, the most suitable carbon source and its optimal concentration should be chosen to establish the efficient production process

of useful metabolites. These factors depend on plant species and products, therefore it is necessary to optimize the medium compositions including carbon sources in each case. From an economical point of view, the use of more inexpensive carbon sources is appropriate in industry and crude sugars such as molasses have been examined.

Table 3. – Media for Plant Tissue and Cell Cultures (mg/L)

Components	Murashige-Skoog (1962)	White (1963	Gamborg (1968)	Nitsch (1951)	Heller (1953)	Schenk - Hildebrandt (1972)	Nitsch - Nitsch (1967)	Kohlenbach - Schmidt (1975)	Knop (1865)
$(NH_4)_2SO_4$	-	-	134	-	-	-	-	-	-
$MgSO_{4\times}\ 7H_2O$	370	720	500	250	250	400	125	185	250
Na_2SO_4	-	200	-	-	-	-	-	-	-
KC1	-	65	-	1,500	750	-	-	-	-
$CaC1_{2\times}\ 2H_2O$	440	-	150	25	75	200	-	166	-
$NaNO_3$	-	-	-	-	600	-	-	-	-
KNO_3	1,900	80	3,000	2,000	-	2,500	125	950	250
$Ca(NO_3)_{2\times}$ $4H_2O$	-	300	-	-	-	-	500	-	1,000
NH_4NO_3	1,650	-	-	-	-	-	-	720	-
$NaH_2PO_{4\times}$ H_2O	-	16.5	150	250	125	-	-	-	-
$NH_4H_2PO_4$	-	-	-	-	-	300	-	-	-
KH_2PO_4	170	-	-	-	_	-	125	68	250
$FeSO_{4\times}\ 7H_2)$	27.8	-	27.8	-	-	15	27.85	27.85	-
Na_2EDTA	37.3	-	37.3	-	-	20	37.25	37.25	-
$MnSO_{4\times}\ 4H_2O$	22.3	7	10 (1 H_2O)	3	0.1	10	25	25	-
$ZnSO_{4\times}\ 7H_2O$	8.6	3	2	0.5	1	0.1	10	10	-
$CuSO_{4\times}\ 5H_2O$	0.025	-	0.025	0.025	0.03	0.2	0.025	0.025	-
H_2SO_4	-	-	-	0.5	-	-	-	-	-
$Fe_2(SO_4)_3$	-	2.5	-	-	-	-	-	-	-
$NiC1_{2\times}\ 6H_2O$	-	-	-	-	0.03	-	-	-	-
$CoC1_{2\times}\ 6H_2O$	0.025	-	0.025	-	-	0.1	0.025	-	-
$A1C1_3$	-	-	-	-	0.03	-	-	-	-
$FeC1_{3\times}\ 6H_2O$	-	-	-	-	1	-	-	-	-
$FeC_6O_5H_{7\times}$ $5H_2O$	-	-	-	10	-	-	-	-	-
K1	0.83	0.75	0.75	0.5	0.01	1.0	-	-	-
H_3BO_3	6.2	1.5	3	0.5	1	5	10	10	-
$Na_2M_0O_{4\times}$ $2H_2O$	0.25	-	0.25	0.25	-	0.1	0.25	0.25	-
Sucrose Glucose	30,000 -	20,000 -	20,000 -	50,000 or 36,000	20,000 -	30,000 -	20,000~30,000 -	10,000 -	- -
Myo-Inositol	100	-	100	-	-	1,000	100	100	-
Nicotinic Acid	0.5	0.5	1.0	-	-	0.5	5	5	-
Pyridoxine HC1	0.5	0.1	1.0	-	-	0.5	0.5	0.5	-

Thiamine HC1	0.1-1	0.1	10	1	1	5	0.5	0.5	-
Ca-Pantothenate	-	1	-	-	-	-	-	-	-
Biotin	-	-	-	-	-	-	0.05	0.05	-
Glycine	2	3	-	-	-	-	2	2	-
Cysteine HC1	-	1	-	10	-	-	-	-	-
Folic Acid	-	-	-	-	-	-	0.5	0.5	-
Glutamine	-	-	-	-	-	-	-	14.7	-

Vitamins

The basal media described above such as MS medium include myo-inositol, nicotinic acid, pyridoxine HCl and thiamine HCl. Among these vitamins, thiamine is an essential one for many plant cells and other vitamins stimulate the growth of the cells in some cases. The level of myo-inositol in the medium is 100 mg/L which is very high although it is not clear whether such a high level of the vitamin is required.

Phytohormones

Phytohormones or growth regulators are required to induce callus tissues and to promote the growth of many cell lines. As an auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) or naphthaleneaceic acid (NAA) is frequently used. The concentration of auxins in the medium is generally between 0.1 to 50 µM. Kinetin or benzyladenine as a cytokinin is occasionally required together with auxins for callus induction at concentrations of 0.1 to 10 µM. Other derivatives of auxin and kinetin are also used in some cases. Since each plant species requires different kinds and levels of phytohormones for callus induction, its growth and metabolites production, it is important to select the most appropriate growth regulators and to determine their optimal concentrations. Gibberellic acid is also added to the medium if necessary.

Organic Supplements

In order to stimulate the growth of the cells, organic supplements are sometimes added to the medium. These supplements include casamino acid, peptone, yeast extracts, malt extracts and coconut milk. Coconut milk is also known as a supplier of growth regulators.

Methods

Preparation of Media

To prepare the medium, many researchers mix the stock solutions which were made previously since the medium compositions are generally complicated.

For example, MS medium is prepared as follows:

a. MS-Micronutrient stock solution (store in freezer)

Ingredient	mg/100 ml
H_3BO_3	620
$MnSO_4\ 4H_2O$	2230
$ZnSO_4\ 7H_2O$	860
$Na_2MoO_4\ 2H_2O$	25
$CuSO_4\ 5H_2O$	2.5
$CoCl_2\ 6H_2O$	2.5

b. Vitamins (store in freezer)

Vitamins	mg/100 ml
Nicotinic acid	100
Thiamine HCl	1,000
Pyridoxine HCl	100
Myo-Inositol	10,000

c. Calcium chloride

$CaCl_2\ 2H_2O$	15 g/100 ml

d. Potassium iodide (store in amber bottle in refrigerator)

KI	75 mg/100 ml

e. 2,4-D (2.2 mM)

Dissolve 50 mg 2,4-D in 2 to 5 ml ethanol, heat slightly and gradually dilute to 100 ml with water (Store in refrigerator).

f. NAA (2.8 mM)

Prepare the same as 2,4-D above.

g. Kinetin (1 mM)

Dissolve 21.5 mg of kinetin in a small volume of 0.5 N HCl by heating slightly and gradually diluting to 100 ml with distilled water (Store in refrigerator). Similar procedures can be used for other cytokinins.

A certain volume of each stock solution is mixed and an appropriate carbon source is added to the mixture. After pH is adjusted to around 5.5 with 0.2 N KOH or 0.2 N HCl, distilled or deionized water is added to the mixture up to the certain volume required. Agar (0.6 to 1.0% wt/vol) is added for a solid medium.

The medium thus prepared is distributed into vessels such as Erlenmyer flasks (for example, 50 ml of the medium in a 300 ml volume Erlenmyer flask) and sterilized by using an autoclave at 120° C for 15 minutes. The sterilization conditions should be varied based on the volume of the medium and the size of the vessel.

Callus Induction

Explants are sterilized with 2% sodium hypochlorite solution and/or 70% ethanol solution. The period of time for submerging the plant materials in these solutions depends upon plant species, their parts and age. For example, a piece of stem of tobacco plant (approximately 3 cm in length) is submerged in 70% ethanal solution for 2-3 minutes and then in 1.2% sodium hypochlorite solution for 10 minutes. The explants should be rinsed with sterilized water.

The stem or any other part of plants thus sterilized is cut to approximately 1 cm in length using a sterilized scalpel and each piece is transferred with tweezers to a solid medium in a flask or a petri-dish. The plant material is incubated aseptically at around 25° C on the solid medium for several weeks or more and a callus is produced. The callus is subcultured by transferring a small piece to fresh solid medium. After several subsequent transfers, the callus becomes soft and fragile.

Suspension Culture

The growth rate of the suspension cultured cells is generally higher than that of the solid culture. The former is more desirable particularly in production of useful metabolites in a large-scale. A piece of the callus is transferred to a liquid medium in a vessel such as an Erlenmyer flask and the vessel placed on a rotary or reciprocal shaker. The culture conditions depend on plant species and other factors, but in general, the cells are cultivated at 100 r.p.m. on a rotary shaker at 25° C; some researchers are fond of much slower, or faster speeds. By subculturing for several generations, a fine cell suspension culture containing small cell aggregates and single cells is established. The time required to establish the cell suspension culture varies greatly and depends on the tissue of the plant species and the medium composition. The cells in suspension are also used for a large-scale culture with jar-fermentors and tanks.

Scaling-up

For commercialization, it is necessary to progress through several stages increasing the volume at each stage until the requisite bioreactor size is attained. In theory, it is anticipated that such large scale suspension cultures will be suitable for industrial production of useful plant chemicals such as pharmaceuticals and food additives, in a manner similar to that of microbial fermentation. Generally speaking, the culture period in plant cell cultures is longer than that in microbial cultures, and it is crucial to protect against microbial contamination.

Equipment and Facilities

Laboratory

There are many textbooks describing very sophisticated laboratory systems for plant cell cultures. However, it is not always necessary to design special laboratories for this technology, but general microbiology laboratories can be used, although aseptic conditions are a prerequisite for incubation of plant cells as well as microbial cultures.

The following equipment is required:

Laminar air flow cabinets: The cabinets are commercially available in different sizes. They are placed in the laboratory where needed. If there is a sterile room, the cabinets are not always necessary.

Autoclave: Autoclaves in different sizes are commercially available.

Oven for dry sterilization: Although autoclaves can be used for dry sterilization, an oven is useful for sterilization of scalpels and glass-wares such as petri-dishes, pipets and others.

Equipment for sterilization by filtration: The medium containing carbon sources and growth regulators are simultaneously sterilized using a autoclave but sometimes aseptic filtration is favorable to avoid decomposition of unstable chemicals. The equipment is also commercially available.

Water distillation apparatus or pure water demineralizer: To prepare media, distilled water or deionized water is generally used although tap water can be used particularly in large-scale cultivation of plant cells in a large fermentor from an economical point of view.

Culture rooms and/or Cabinets: To cultivate the plant cells, culture rooms under different temperature or and/or cabinet-type incubators are essential facilities. Temperature and light intensity as well as a duration of lighting in the room and/or in the cabinet are controlled under the optimal conditions.

Shelves: Shelves built from rigid wire mesh to allow maximum air movement and minimize shading should be used in the culture room.

Shakers: A rotary shaker or a reciprocal shaker is necessary for suspension cultures.

Fermentors or Bioreactors

In order to cultivate plant cells in a large-scale, fermentors with different sizes are useful. Various types of fermentors have been designed by many researchers since the end of 1950's as seen in Fig. 1. The most simple vessel is a carboy system described by Tulecke and Nickell in 1959 which consists of a rubber-stoppered 20 L carboy fitted with four tubes (air-in, air-out, medium-in and sample-out). Filtered compressed air is employed for oxygen supply, aeration and agitation of the medium. A roller-bottled system using a round flask was used by Lamport in 1964. A V-shape fermentor was proposed by Veliky and Martin. It is an inverted flask carrying two teflon-coated stirring bars on a glass pin situated at the bottom of the flask. A drain/sample port is also located at the bottom. The top of the flask is fitted with four standard taper penetrations.

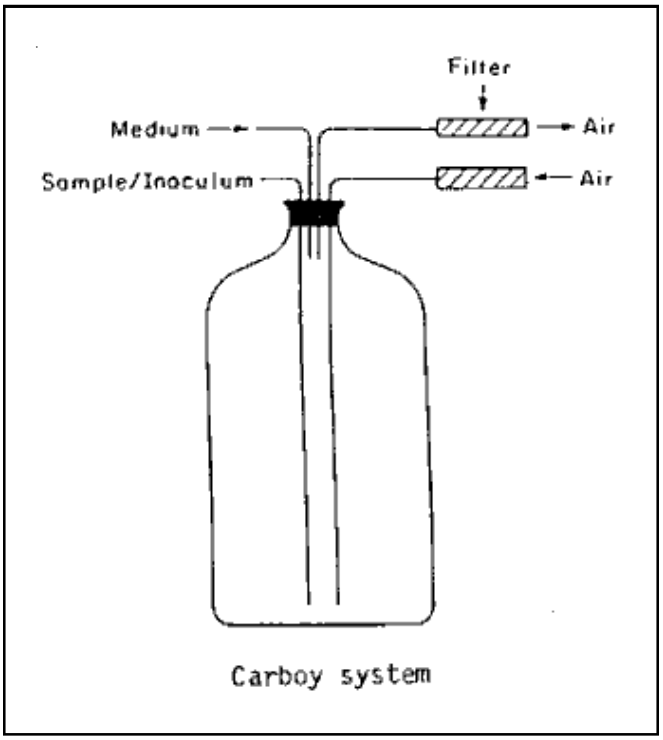
However, the most common types of system on the bench is a stirred-jar fermentor which is used for microbial cultivation although some minor alteration is made. For example Martin et al. increased the size of each impeller blade to 1 inch with a commercially available 7.5 L New Brunswick Microferm Fermentor.

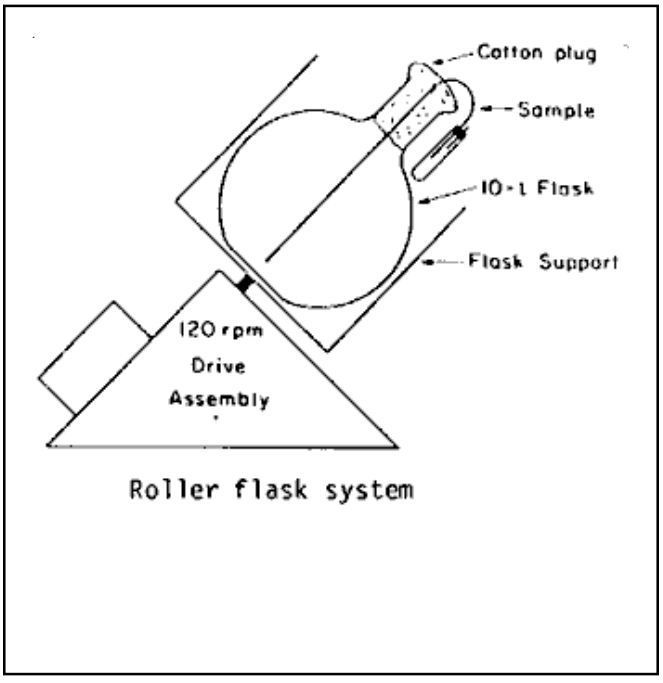
Kato et al.suggested that an agitation speed of 50 to 100 r.p.m. was most appropriate for the growth of tobacco cells in stirred-jar fermentors. It is true that cultured plant cells are more fragile than bacterial cells, however, Martin noted: "it seems obvious that cell lines differ in their resistance to shear effects and that a single optimum agitation speed cannot be designed for all lines".

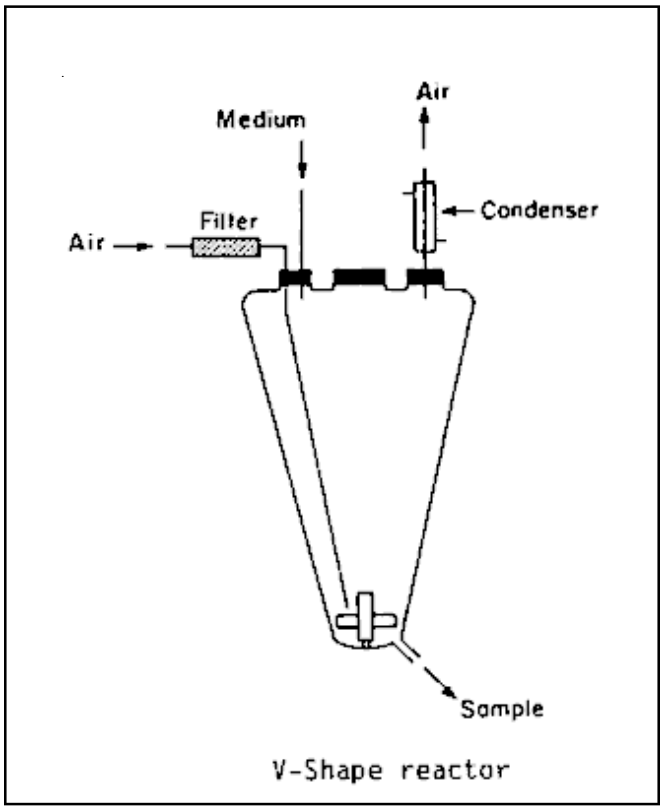
Wagner et al. compared a variety of fermentor types equipped with different agitation and aeration systems with various productivities of cell mass and anthraquinones using Morinda citrofolia cells, and recommended the air-lift type fermentor as the most suitable system (Fig.2). Tanaka et al. designed a rotary-drum type fermentor having an in-let and an out-let at the side of the fermentor (Fig. 4). The fermentor itself rotates slowly like as a rotary bottle. Recently, Ten Hoopen et al.discussed the

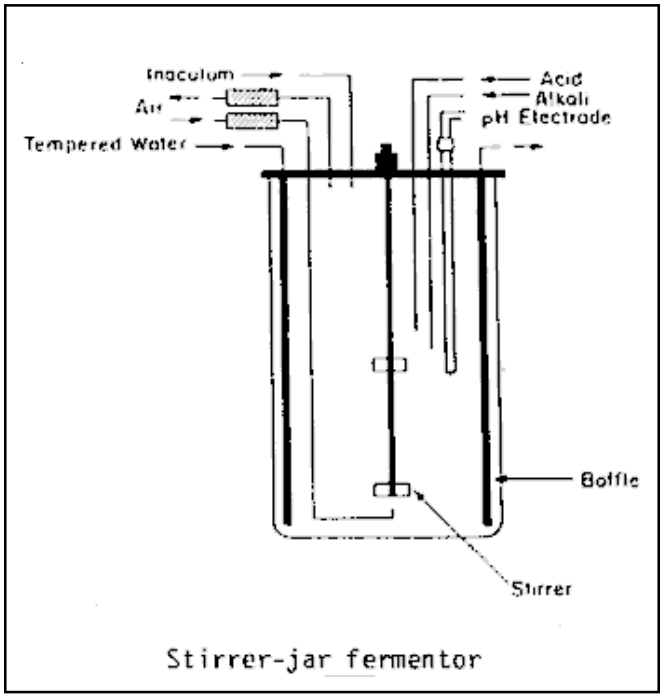
problems and profiles of large-scale plant cell culture. However, fermentors installed with agitators which are similar to those of microbial culture have been employed for commercial production of shikonin and ginseng cells although some modification in equipment was made to optimize physical conditions.

A company in Germany, DIVERSA, has equipped 5 sophisticated fermentors of up to 75,000 L for plant cell cultures. Although detailed modifications to those fermentors has not been disclosed, the photos show that they are similar to ordinary microbial fermentors. The company has cultivated Echinacea purpurea cells for the manufacture immunobiologically active polysaccharides.



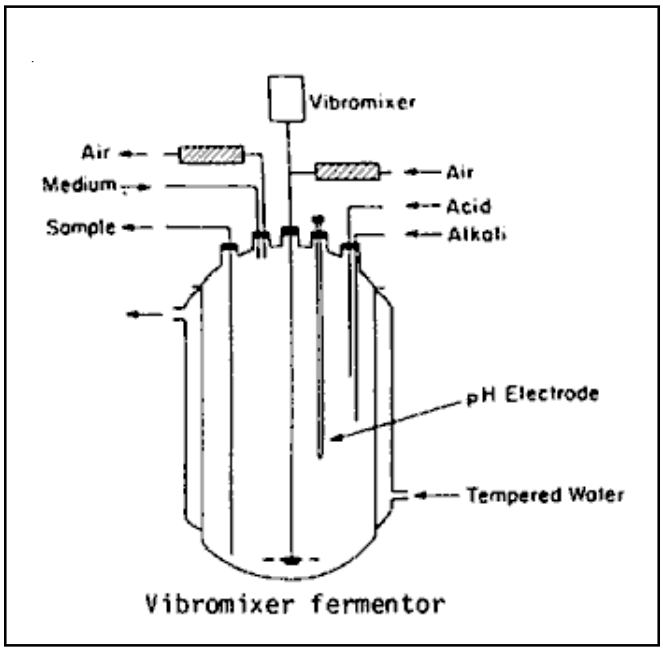


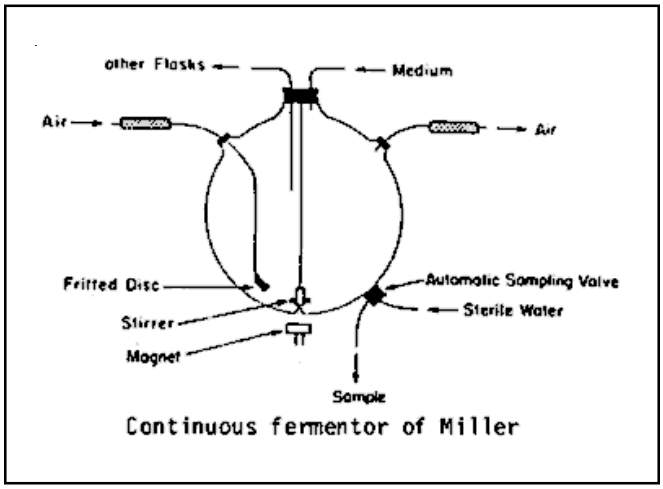


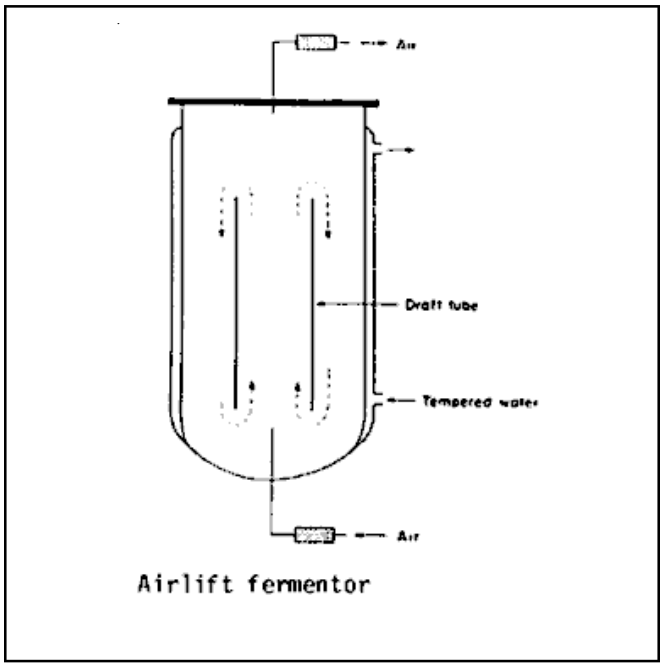


Source: Martin, S.M., In "Plant Tissue Culture as a Source of Biochemicals" Ed. Staba, E.J. P. 151-164 (1980). CRC Press, Florida, USA

Figure 1. Various Fermentors for Plant Cell Cultures







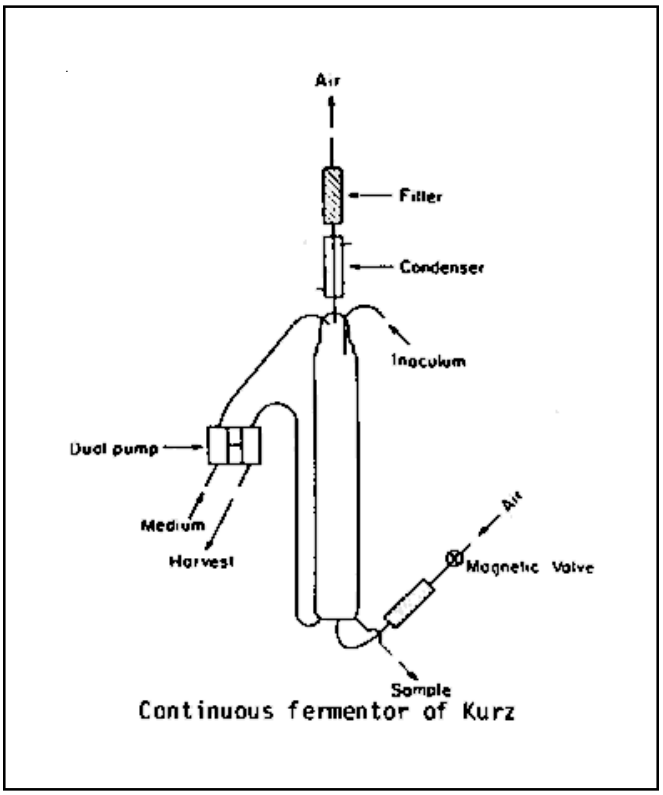
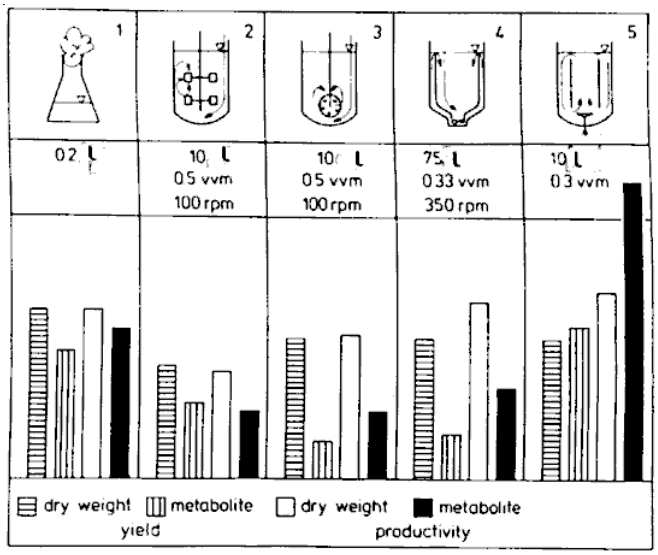


Figure2. (Continued) Various Fermentors for Plant Cell Cultures



Source: Wagner, F. In "Plant Tissue Culture and Its Bio-technological application"Ed. Barz, W. et al., p. 250 (1977). Springer-Verlag, Berlin Heidelberg.Figure3. Comparison of Yeld and Productivity for Cell Mass and Anthraquinones in Varoius Reactor Systems1. Shake flask; 2. flat blade turbine; 3. perforated disk impeller; 4. draft tube reactor with kaplan turbine; 5. airlift reactor

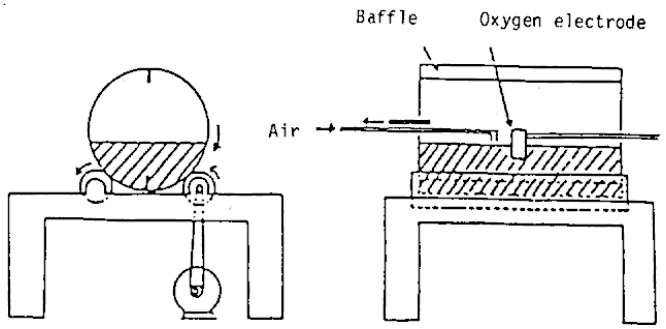


Figure 4. Rotating Drum Fermentor Designed by Tanaka

Tanaka, H., et al., Biotechnol. Bioeng., 24 2359 (1983)

Large Scale Cultures

Commercial applications of tissue culture necessitate scaling up of culture systems; this can be achieved by (i) increasing the number or (ii) size of culture vessels, or (iii) a combination of both. The strategy of scaling up depends chiefly on the physical condition of medium, i. e., agar or liquid, used for the cultures.

Agar Cultures

Agar-gelled medium is the most commonly used for experimental and some commercial activities, e.g., micropropagation. Generaliy during experimentation, culture tubes of 25 x 150 mm are used for culture; here scaling up can be achieved by increasing the number of culture vessels. These culture tubes are usually arranged in test tube baskets or stands which are placed on culture racks. Further scaling up is afforded by the use of larger culture vessels, e.g., 38 x 200 mm culture tubes and, more particularly, 500 ml wide mouth bottles, magenta boxes etc. In case of bottles/ magenta boxes 2-4 separate pieces of the tissue inoculum may be placed in a single vessel. Half litre bottles are the most commonly used culture vessels in commercial micropropagation activities. Scaling up of agar cultures involves considerable labour since each vessel has to be handled individually during subculture etc.

Suspension Cultures

Use of liquid medium in suspension cultures allows easy and extensive scaling up by employing bioreactors, although a limited scaling up can be achieved by using larger culture flasks (usually 250 ml flasks having 50 ml medium are used).

What is a Bioreactor?

A bioreactor is a culture vessel, generally of a large volume, e.g., 1L to over 1000L which has provisions for:

i. aeration,

ii. stirring to achieve medium and cell mixing,

iii. contamination control and

iv. replacement of used medium and/or used medium plus cells.

The bioreactors used for culture of plant cells may be of following 4 types: (1) batch bioreactors, (2) continuous bioreactors, (3) multistage bioreactors and (4) immobilized cell bioreactors; all bioreactors, except the last one are commonly called stirred tank reactors since the vessel has a device for stirring.

Batch Bioreactors. In such bioreactors, the medium and inoculum are loaded in the beginning and the cells are allowed to grow. There is no additional replacement of medium, and the entire cell mass is harvested at the end of incubation period. The characteristic features of such bioreactor systems are as follows:

i. continuous depletion of medium,

ii. accumulation of cellular wastes,

iii. alterations in growth rate, and

iv. continuous change in the composition of cells. The spin filter bioreactor (Fig. 5.) can be used as a batch bioreactor by closing the inlet for medium and the outlets for medium/ medium plus cells.

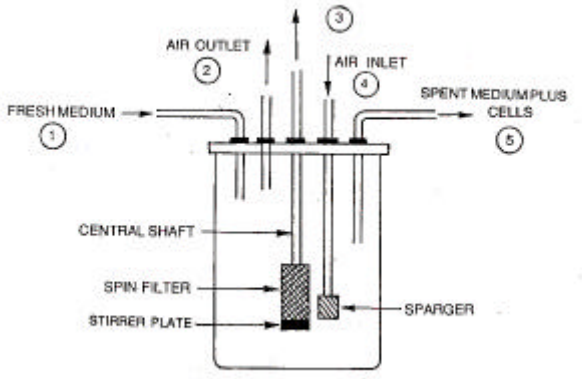


Fig. 5. A schematic representation of a spin-filter bioreactor.

The bioreactor has ports for (1) addition of fresh medium, (2) air space, (3) removal of only spent medium (minus cells; they are separated by the spin-filter) through the hollow central shaft, (4) entry of air to the sparger, and (5) removal of spent medium plus cells. When all the 5 ports are in operation, the bioreactor is of continuous flow type. Burt when only ports (2) and (4) (air outlet and inlet, respectively) are open [the remaining ports, i.e. (1), (3) and (5) are closed], the bioreactor becomes a batch type.

Continuous Bioreactors. In such bioreactors, there is continuous inflow of fresh medium and outflow of used medium (with or without cells) during the entire incubation period. A spin-filter bioreactor is a good example of continuous flow bioreactor; it has the following features.

1. The central shaft of bioreactor houses a spinning filter which enables the removal of used medium free of cells, through the shaft.
2. A stirrer plate magnetically coupled to the central shaft provides continuous stirring; the spinning filter also stirs the culture
3. The culture is aerated by a sparger which allows a wide range of aeration rates
4. A port is provided for addition of fresh medium, while
5. Another port enables removal of the culture (used medium + cells) as per need.

This bioreactor provides a highly versatile system for control on medium change rate and on cell density; this becomes possible due to the two routes for medium removal while only one of them allows the removal of cells. A continuous flow bioreactor is used to grow cells at a specified cell density in an active growth phase. Such cultures may either provide inoculafor further culture or may serve as a continuous source of biomass yields.

Multistage Bioreactors. Such culture systems use two or more bioreactors in a specified sequence each of which carries out a specific step of the total production process (Fig.6).

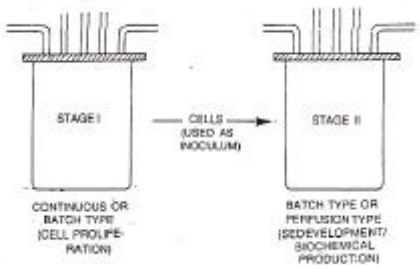

STAGE I
CELLS (USED AS INOCULUM)
STAGE II
CONTINUOUS OR BATCH TYPE (CELL PROLIFERATION)
BATCH TYPE OR PERFUSION TYPE (SE DEVELOPMENT/ BIOCHEMICAL PRODUCTION)

Fig. 6. A two-stage bioreactor configuration using two spin-filter bioreactors.

A perfusion type bioreactor allows regular circulation or change of medium; this is achieved by regularly removing the used medium only through the spin-filter [port (3); Fig. 5] and adding fresh medium through the medium port [port (1)]. A perfusion type bioreactor also allows change of one type of medium by another as per need of culture system.

The simplest situation would involve 2 bioreactors. For the production of a biochemical like shikonin, both the bioreactors are batch type: the first bioreactor provides conditions for rapid cell proliferation and favours biomass production, while the second bioreactor has conditions conducive for shikonin biosysnthesis and accumulation. The cell biomass is collected from the first stage bioreactor and is used as inoculum for the second stage reactor. Similarly, for large scale somatic embryo (SE) production, the first reactor may be in continuous mode, while the second may be of batch type. The continuous first stage reactor provides the conditions necessary for rapid proliferation of embryogenic cells (but not for SE development). The cell mass from this bioreactor serves as a continuous source of inoculum for the second stage batch type bioreactor which has conditions necessary for embryo development and maturation (but not for cell proliferation).

The use of continuous first stage bioreactor (i) avoids the time, labour and cost needed for cleaning etc. of a batch reactor between two runs, (ii) eliminates the lag phase of batch cultures, and (iii) provides a more homogeneous and actively growing cell population. However, continuous cultures, due to their long run periods, have a greater contamination risk than batch bioreactors.

Immobilized Cell Bioreactors: These bioreactors are based on cells entrapped either in gels, such as, agarose, agar, chitosan, gelatine, gellan, polyacrylamide and calcium alginate to produce beads, or in a membrane or metal (stainless steel) screen compartment or cylinder.A simplified version of a device based on metal screen is shown in (Fig. 7). The membrane/screen cylinder containing cells is kept in a chamber through which the medium is circulated from a recycle chamber. The medium flows parallel to the screen cylinder and diffuses across the screen into the cell mass. Similarly, products from cells diffuse into the medium and out of the screen cylinder. The membrane/screen compartment housing the cells may be cylindrical or flat, and medium movement may so adjusted as to flow across the screen compartment rather than parallel to it. The technology is being refined for commercialization. Fresh medium is regularly added to and equivalent volume of used medium is withdrawn from the recycling chamber to maintain its nutrient status.

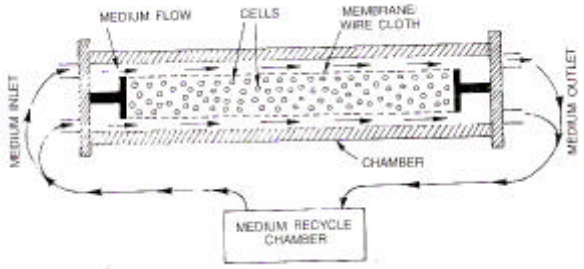


Fig. 7. A schematic representation of a simple assembly to serve as immobilized cell bioreactor.

Cell immobilization changes the physiology of cells as compared to that of cells in suspension. This technique is useful where the biochemical of interest is excreted by the cells into the medium. Product excretion may also be brought about by immobilization itself, or by certain treatments like altered pH, use of DMSO (dimethyl sulfoxide) as a permealizing agent, changed ionic strength of medium, an elicitor etc. Immobilized cell reactors have the following advantages: (i) no risk of cell wash out, (ii) low contamination risk, (iii) protection of cells from liquid shear, (iv) better control on cell aggregate size, (v) separation of growth phase (in a batch/continuous bioreactor) from production stage (in an immobilized cell bioreactor), (vi) cellular wastes regularly removed from the system, and (vii) cultures at high cell densities.

Problems in Large Scale Culture of Plant Cells

Large scale culture of plant cells for commercial application like biochemical, artificial seed etc. production presents several problems which are summarized below.

1. Plant cells have much slower growth rates than bacteria and fungi, therefore, larger reactors and longer fermentation times are necessary.
2. The long fermentation time increases the risk of contamination
3. Plant cells are rather sensitive to shear. Therefore, fermenters with conventional mechanical stirring are not suitable for their culture. Bioreactors having specially designed mechanical stirrers or airlift fermenters are far more suitable.
4. Plant cells show cytogenetic, genetic and epigenetic variations during culture. Therfore, the characteristics of a cell

population may chage during culture, and prolonged continuous cultures may not be desirable.

5. In case of biochemical production, often conditions favouring rapid growth may suppress biochemical formation and vice versa. In general, organized cell masses and cells under stress are more likely to produce useful biochemicals. In all such cases, therefore, a multistage bioreactor system should be employed.

Conclusion

Some of the techniques like, biochemical production require continuous in vitro culture of cells for product isolation. In such cases, a scaling up of the culture operations becomes essential. Scaling up may often utilize fermenters which involve engineering disciplines like process engineering etc. This Lesson gives a basic idea on how to obtain callus and suspension cultures in vitro for product isolation and also a general information on the types of fermenters used for commercial scaling up of culture systems.

Questions

1. Give a brief historical background of the scaling up of production of plant secondary metabolites.
2. Write short note on the types of equipments (fermenters) used.
3. Give an account of the bioreactors used for scaling up of cultures.

Note